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SPECIFIC AFFINITY LABELING OF μ OPIOID RECEPTORS IN RAT BRAIN BY *S*-ACTIVATED SULFHYDRYLDIHYDROMORPHINE ANALOGS

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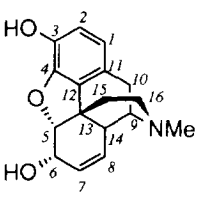
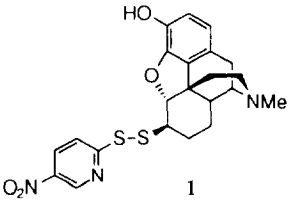
Abstract *S*-Activated sulfhydryldihydromorphine analogs **1** and **2** were synthesized. In the rat brain receptor binding assays, both **1** and **2** exhibited considerably high affinities for μ opioid receptors (IC_{50}; **1**= 31.1 nM, **2**= 10.7 nM). However, when each analog was incubated with membranes for the purpose of getting disulfide bridgings, **1** (EC_{50} = 58 nM) was found to affinity-label the μ receptors about 30 times more effectively than **2** (EC_{50} = 1700 nM).

Introduction

The existence of multiple opioid receptors in the brain and the peripheral tissues has been documented in biochemical and pharmacological studies. Several lines of evidence that indicate the existence of thiol group(s) in receptors of neurotransmitters and neuropeptides have been reported, although their functional roles have not yet been clarified. Larsen *et al.* suggested that in opioid receptors there are at least two different types of thiol groups sensitive to *N*-ethylmaleimide.[1] Both of these thiols are originated from the cysteine residues: one is the cysteine β-thiol in the GTP-binding regulatory protein Gi, and the other is in the ligand binding site of receptor protein.

Recently, we have shown that *S*-activated (-)-6β-sulfhydryldihydromorphine (**1**), designed as an affinity-labeling ligand for opioid receptors, is able to bind covalently to a free thiol group in the binding site of the opioid receptors in the peripheral muscle tissues of guinea-pig ileum *via* thiol-disulfide exchange reaction.[2] Such affinity ligands are the potential affinity probe for characterization of opioid receptors. Since the sepcificity of compound **1** to affinity-label the peripheral receptors was not so high, we have prepared in this study *S*-activated (-)-8β-sulfhydryldihydromorphine analog (**2**), expecting the regiochemical effect on predominant affinity labeling of opioid receptors. In the present study in order to evaluate the binding abilities for both δ and





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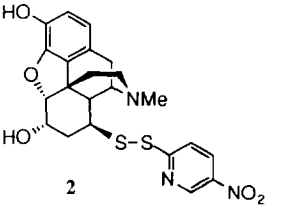

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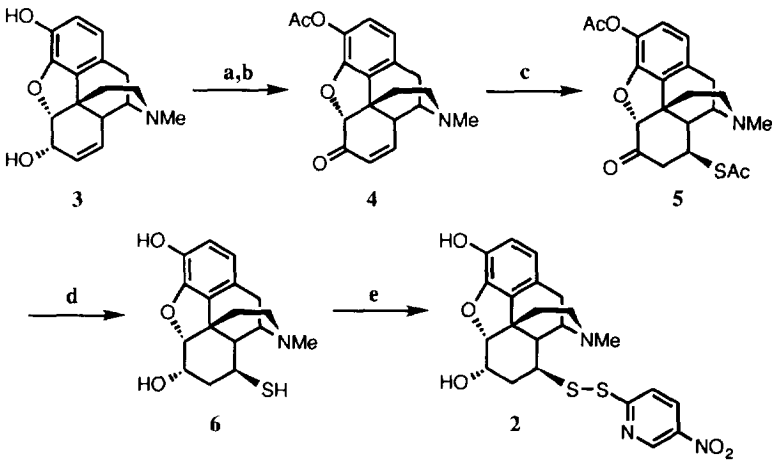
μ receptors, we tested compounds **1** and **2** by using membrane preparations from rat brain.

We here describe their binding affinities and abilities to bind to receptors covalently.

Chemistry

Compound **1** was prepared essentially as reproted previously.[3] The synthesis of compound **2** was shown in Scheme 1. Swern oxidation of 3-acetylmorphine gave the 3-acetyl morphinone (**4**), which was treated with thioacetic acid in the presence of 2,6-lutidine to produce stereoselectively β-thioester isomer (**5**). Then the thioester (**5**) was treated with excess sodium borohydride in EtOH to give a mixture of epimers quantitatively (6α : 6β = 7 : 3). The 6α epimer (**6**) was separated from a mixture by column chromatography in 65% yield. Finally, 8β-sulfhydryldihydromorphine (**6**) was treated with 5-nitro-2-pyridinesulfenyl chloride in dichloromethane at 0 °C to afford 8β-(5'-nitro-2'-pyridyldithio) dihydro morphine (**2**) in 92% yields.

Scheme 1



Reagents: a) Ac_2O, $NaHCO_3$ (100%); b) DMSO, trifluoroacetic anhydride, CH_2Cl_2, -60 °C; Et_3N (98%); c) AcSH, 2,6-lutidine, benzene (79%); d) $NaBH_4$, EtOH (65%) e) 5-nitro-2-pyridinesulfenyl chloride, CH_2Cl_2, 0 °C (92%).

Opioid Receptor Binding

Specific binding affinities of compounds **1** and **2** for μ opioid receptors in rat brain were determined by evaluating their ability to displace [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]DAGO).[4] The radio-ligand receptor binding assays were carried out as described previously.[5] Figure 1A shows the dose-response curves analyzed by the computer program ALLFIT, which constructs the least-square estimates of the logistic curves relating binding of labeled ligand to concentrations of unlabeled ligand.[6] It is clear that both **1** and **2** are considerably potent to interact with μ receptors. The IC_{50} values, the halfmaximal concentration of unlabeled ligands for inhibition of binding of labeled ligand, were 31.1 nM and 10.7 nM for **1** and **2**, respectively. Binding affinity for δ opioid receptors was evaluated by using [^{3}H]-[D-Ser^2,Leu^5]enkephalyl-Thr^6 ([^{3}H]DSLET) as a tracer.[7] Compound **1** exhibited the IC_{50} value of 206 nM. The calculated value = ca. 7 of μ/δ selectivity ratio indicated that **1** is about 7 times more preferential to μ recptors than to δ receptors.

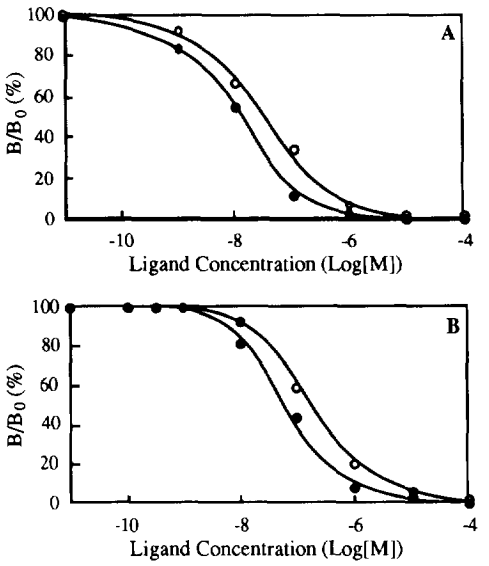


Figure 1. Dose-responce curves of *S*-activated ligands in rat brain membrane preparations using [^{3}H]DAGO for μ opioid receptors (A), and [^{3}H]DSLET for δ opioid receptors (B). Compound **1** (O—O) and compound **2** (●—●). B/B_0 (%) = relative binding activities (%) of the remained radio-labeled ligand over total specific binding.

Compound **2** exhibited the IC_{50} value of 67.9 nM for δ opioid receptors, indicating that it is also μ-selective (μ/δ ratio > 6).

The ability of compounds **1** and **2** to label irreversiblly opioid receptors can be examined by their incubation with membranes and subsequent assays for the biological activity or the receptor binding. Since the assessment of biological activities usually requires to evaluate the efficacy between receptor binding and activity, in the present study the capability of morphine analogs for irreversible-labeling was examined by the radio-ligand receptor binding assays. When **1** or **2** was incubated with rat brain membranes, they would first bind to the ligand binding site of the receptors. However, if the thiol group is present near the ligand bound in the receptor, the activated SH group of ligand will react with this free thiol, resulting in the formation of a covalent disulfide bond. Such affinity labeling of receptors would substantially reduce the number of receptors available for binding of the ligands added afterwards. Thus, after incubation of membranes with *S*-activated ligands, the ordinary receptor binding assay would estimate the amount of receptors unlabeled and consequently the amount of labeled receptors. In order to estimate the total amount of the free receptors, the amount of DAGO-enkephalin that displaces radio-labeled [^{3}H]DAGO was measured.[8] This binding assay evaluates the extent of affinity labeling of μ receptors, because DAGO binds exclusively to the μ receptors. For δ opioid receptors, to estimate the total amount of the free receptors, the amount of DSLET that displaces radio-labeled [^{3}H]DSLET was measured.

Affinity labeling experiment was carried out essentially as described previously.[8] Rat brain membranes in 10 mM Tris-HCl buffer (pH 7.5) were incubated with *S*-activated ligands (0.2 nM—20 μM) or without ligands (control) in the presence of bacitracin (100 μg ml^{-1}) at 25 °C for 30 min. Membranes were then centrifuged (40000 g, 15 min) and suspended in the same volume of buffer to homogenize with Polytoron

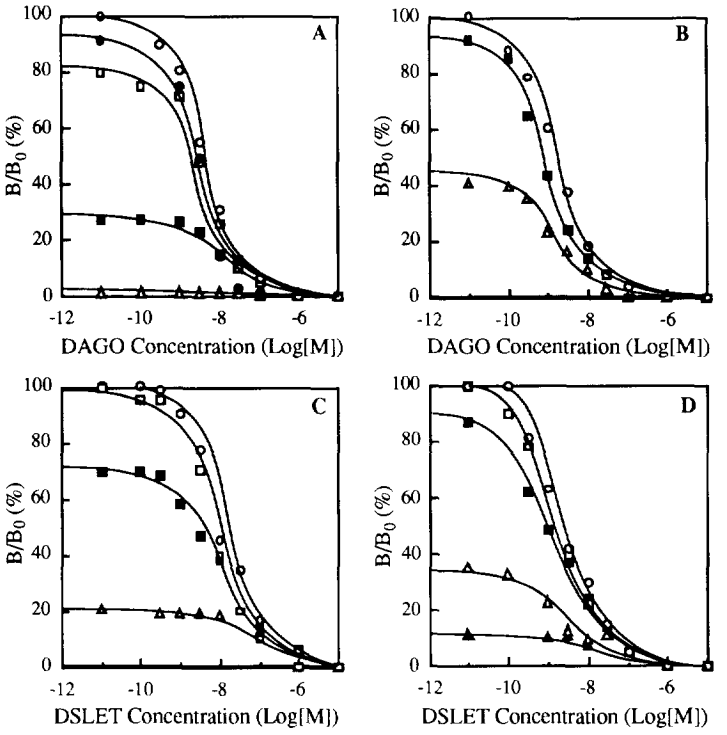


Figure 2. Dose-responce curves of DAGO or DSLET in rat brain membrane preparations using [^{3}H]DAGO for μ opioid receptors, and [^{3}H]DSLET for δ opioid receptors. Rat brain membrane was incubated with 6*S*-activated ligand (**1**) before μ binding assays (A) and δ binding assays (C). Incubation were carried out with 8*S*-activated ligand (**2**) before μ binding assays (B) and δ binding assays (D). The concentrations of *S*-activated ligands (**1** or **2**) are (2nM, ●—●), (20nM, □—□), (200nM, ■—■), (2μM, △—△), (20μM, ▲—▲), (control, ○—○).

homogenizer, then incubated at 25 °C for 15 min. These washing operations were repeated to remove completely the ligands bound nonspecifically to membranes, and at least five times washings were required (data not shown). Washed membranes were finally assayed for radio-ligand receptor binding assays. When **1** was incubated with rat brain membrane, it was found that the amount of free receptors diminished sharply, depending upon the concentrations of **1** (Fig. 2A).

When the extents (%) of affinity-labeling, which were calculated on the basis of assay results shown in Figs. 2A and 2B, were plotted against the concentrations of *S*-activated ligands incubated, the typical sigmoidal curves were depicted as shown in Fig. 3. Figure 3A indicates, for exapmle, that when the rat brain membrane is incubated with 100 nM of the 6β-*S*-activated ligand **1**, it occupies approximately 70% of μ receptors, while 8β-*S*-activated isomer **2** cross-links only about 3% of μ receptors. The effective concentration (EC_{50}) to occupy the half-maximal amount of receptors was estimated from Fig. 3A, and those were 58.1 nM and 1700 nM for compounds **1** and **2**, respectively. It should be noted that **1** is about 30 times more efficient to affinity-label

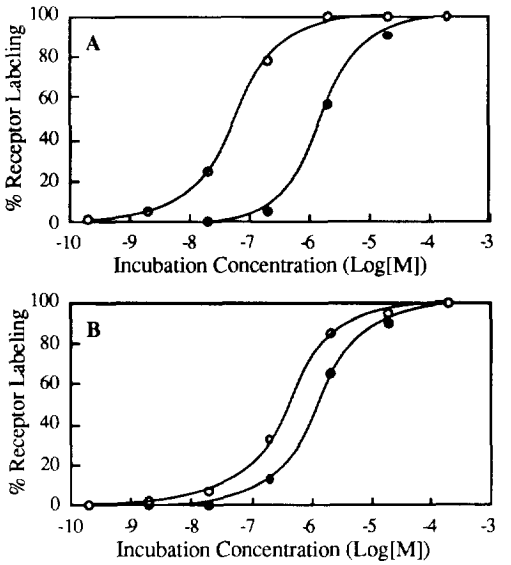


Figure 3. The concentration-dependent affinity labeling of μ (A) and δ (B) opioid receptors by *S*-activated ligands. Compound **1** (O—O) and Compound **2** (●—●).

the μ receptors than its 8*S*-analog **2**.

From Figs. 2C and 2D, the concentration-dependent curves of affinity-labeling of δ receptors was depicted as in Fig. 3B. The EC_{50} values were 402 nM and 1200 nM for compounds **1** and **2**, respectively. Since the IC_{50} and EC_{50} values of compound **1** for μ receptors are almost comparable to each other (31.1 and 58.1 nM, respectively), it appears that the molecule **1** bound to the binding site almost inevitably forms a cross-link with the receptor molecule by a disulfide bonding. Furthermore, the fact that **1** can label the μ receptors about 7 times more selectively than δ receptors should be noted, since this selectivity ratio is compatible to that shown for binding affinities (see above). The structure of 6β-*S*-activated ligand **1** appears to fit preferentially the binding site of μ receptors in which the thiol group is located close to 6β-*S*-activated thiol of compound **1**.

In sharp contrast, compound **2** lacks the potential ability to affinity-label both μ and δ receptors. Particularly, in spite of high binding affinity for μ receptors, its ability to form a cross-link with the receptor molecule was found to be extremely weak. This is certainly the reflection of regiochemistry of thiols activated by the 5-nitro-2-pyridinesulfenyl group, which is attached to position C_6 or C_8. The 8β SH group of **2** is in much less favored regiochemistry to interact with the receptor thiol group.

The receptor thiol group seemed to be present near the portion where the morphine 6-substituent located. Recently, we reported the molecular model building of multiple opioid receptor subtypes.[9] The models indicated that the Cys residues (μ: Cys-321; δ: Cys-303; κ: Cys-315) in the transmembrane VII could participate in ligand-binding (Fig. 4). In the model of the μ-subtype receptor, when **1** was manually docked into the model, the thiol group of Cys-321 was found to be close enough to the activated disulfide bond of **1** (data not shown). The spatial proximity is expected to cause a thiol-disulfide exchange reaction between compound **1** and receptor thiol. This may account for the present results.

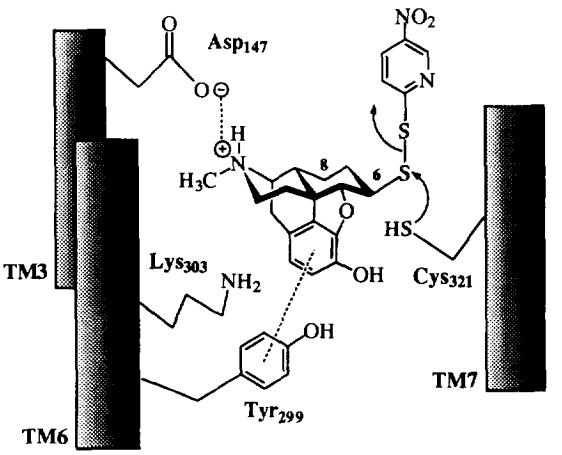


Figure 4. Schematic representation of the interaction between μ-opioid receptor recognition sites and *S*-activated ligand.

Although the role of thiol group in the molecular mechanism of receptor responses has not been clarified yet, the present results together with these obtained previously indicate that the opioid receptor protein contains a distinct free thiol group in the ligand binding site. Attempts to determine the specific cysteine residue are in progress in our laboratories.

Acknowledgment

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